Molecules and Cells

The *Arabidopsis* Phytocystatin AtCYS5 Enhances Seed Germination and Seedling Growth under Heat Stress Conditions

Chieun Song[1], Taeyoon Kim[1,2], Woo Sik Chung[1,2], and Chae Oh Lim[1,2,*]

[1]Systems and Synthetic Agrobiotech Center and PMBBRC, Gyeongsang National University, Jinju 52828, Korea, [2]Division of Life Science, Gyeongsang National University, Jinju 52828, Korea
*Correspondence: colim@gnu.ac.kr
http://dx.doi.org/10.14348/molcells.2017.0075
www.molcells.org

Phytocystatins (PhyCYSs) are plant-specific proteinaceous inhibitors that are implicated in protein turnover and stress responses. Here, we characterized a PhyCYS from *Arabidopsis thaliana*, which was designated AtCYS5. RT-qPCR analysis showed that the expression of *AtCYS5* in germinating seeds was induced by heat stress (HS) and exogenous abscisic acid (ABA) treatment. Analysis of the expression of the β-glucuronidase reporter gene under the control of the *AtCYS5* promoter showed that *AtCYS5* expression during seed germination was induced by HS and ABA. Constitutive overexpression of *AtCYS5* driven by the cauliflower mosaic virus *35S* promoter led to enhanced HS tolerance in transgenic *Arabidopsis*, which was characterized by higher fresh weight and root length compared to wild-type (WT) and knockout (*cys5*) plants grown under HS conditions. The HS tolerance of *AtCYS5*-overexpressing transgenic plants was associated with increased insensitivity to exogenous ABA during both seed germination and post-germination compared to WT and *cys5*. Although no HS elements were identified in the 5'-flanking region of *AtCYS5*, canonical ABA-responsive elements (ABREs) were detected. *AtCYS5* was upregulated in ABA-treated protoplasts transiently co-expressing this gene and genes encoding bZIP ABRE-binding factors (ABFs and AREB3). In the absence of ABA, ABF1 and ABF3 directly bound to the ABREs in the *AtCYS5* promoter, which activated the transcription of this gene in the presence of ABA. These results suggest that an ABA-dependent pathway plays a positive role in the HS-responsive expression of *AtCYS5* during seed germination and post-germination growth.

Keywords: abscisic acid, *cis*-element, gene expression, transcription factor, transgenic plants

INTRODUCTION

Plant cystatins, or phytocystatins (PhyCYSs), are proteinaceous inhibitors of the papain-like (C1A) and legumain (C13) families of plant cysteine proteases (CPs) (MEROPS peptidase database; http://merops.sanger.ac.uk; Christoff et al., 2016). Members of these CP families play many roles in plant growth and development, including seed germination (van der Hoorn 2008), together with the specific endogenous PhyCYSs that regulate CP activity (Szewińska et al., 2016).

The establishment of seed germination and early seedling growth is strongly influenced by various unfavorable environmental conditions, which induce stress responses, therefore negatively affecting these processes. The joint action of papain-like CPs and legumains plays a key role in the degradation of reserve proteins (Zakharov et al., 2004), and their activity is inhibited by PhyCYSs under unfavorable conditions (Julián et al., 2013), including drought (Rodriguez et al., 2010), heat (Je et al., 2014), high alkalinity (Sun et al., 2014), high salinity (Tan et al. 2016), and low temperature stress

Received 12 May, 2017, revised 8 June, 2017; accepted 25 June, 2017; published online 31 July, 2017

eISSN: 0219-1032

(Zhang et al., 2008), as well as osmotic imbalance (Bae et al., 2010), but their complex interactions make it difficult to establish clear connections between a particular stress and the corresponding response at the level of *CP*s and *PhyCYS*s (Kidrič et al., 2014).

The transcriptional regulation of *CP*s and *PhyCYS*s is determined by *cis*-elements located in the promoters of these genes and by the expression of the corresponding transcription factors. The transcripts of several *CP* and *PhyCYS* genes accumulate in imbibed seeds exposed to unfavorable conditions, and the precise control of their expression is important in regulating germination rates in response to stress (Huang and Xu, 2008). For example, the expression of barley *PhyCYS Hv-CPI* in the aleurone layer is regulated by two Dof (DNA-binding one zinc finger) transcription factors (Martínez et al., 2005), and in *Arabidopsis*, two Dof family members, DAG1 and DAG2, have opposite effects on seed germination (Gabriele et al., 2010). *Arabidopsis PhyCYS3* (*AtCYS3*), which is induced by both drought and cold treatment, contains a 9 bp dehydration-responsive element (DRE) in its promoter sequence (Seki et al., 2002). The role of DREs in regulating *PhyCYS* expression is exemplified by *AtCYS3* (synonymous with *AtCYSa*), which is expressed in *Arabidopsis* seedlings exposed to drought, salt, and cold stress (Zhang et al., 2008). The regulation of the *Arabidopsis PhyCYS4* gene, *AtCYS4*, in response to heat stress (HS) occurs via DRE-binding factor 2C (DREB2C), and DREB2C acts as a transcriptional activator of this thermotolerance-related gene during seed germination (Je et al., 2014).

The rate of seed germination is also influenced by the contents of abscisic acid (ABA), which increase in response to various stresses and negatively affect germination and early seedling growth (Verma et al., 2016). ABA-responsive genes, including *PhyCYS*s, have multiple *cis*-elements known as ABA-responsive elements (ABREs) in their promoters. The basic leucine zipper transcription factors (bZIPs) ABRE-binding proteins (AREBs)/ABRE-binding factors (ABFs) can bind to ABRE, resulting in the upregulation of ABA-responsive genes, including *CP*s and *PhyCYS*s (Szewińska et al., 2016). The findings that AREBs and/or ABFs are induced by abiotic stress and that plants overexpressing these factors exhibit enhanced stress tolerance further confirm the importance of these proteins and, hence, ABA in abiotic stress responses (Fujita et al., 2005).

In the present study, we investigated the activity of the *Arabidopsis PhyCYS5* (*AtCYS5*) gene during seed germination and post-germination growth under HS and high ABA conditions. *AtCYS5* expression was modulated by HS and ABA, and plants overexpressing *AtCYS5* exhibited enhanced ABA insensitivity during seed germination and early seedling growth, correlating directly with the ability of *Arabidopsis* plants to tolerate HS. Finally, we showed that *AtCYS5* is transcriptionally regulated and activated by bZIP transcription factors, suggesting that these factors might be components of the ABA signaling pathway from HS to *AtCYS5* induction.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana* L. Heynh wild-type (WT) and transgenic seeds used in this study were in the Columbia (Col-0) background. The seeds were stratified at 4℃ for 3 days in the dark and grown in soil or *in vitro* on phytohormone-free MS medium (MSO; 1% sucrose, 0.25% Phytagel, pH 5.8) at 22℃ under a 16 h light/8 h dark photoperiod with 100 μE m^{-2} s^{-1} light (Song et al., 2016).

A T-DNA insertional mutant containing a single T-DNA insertion in *AtCYS5* (At5g47550) was identified in the SALK T-DNA collection (SALK_149928C). To identify mutants homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and subjected to PCR genotyping using the following *AtCYS5* primer sets: *AtCYS5* P1 forward (5'-TCTAGAATGACTAGTAAGGTCGTCTTCCTT-3'), P2 reverse (5'-AGGTACCAAAGAGCGGTTACATGTTAAAAGC-3'), T-DNA P3 right (5'-TGGGAAAACGGGCGTTACCCAACTT AAT-3'), and P4 left primer (5'-GTGATGGTTCACGTAGTGGG CCATCG-3') (Supplementary Fig. 1A).

Reverse-transcription quantitative PCR (RT-qPCR)

To analyze the expression of *AtCYS5* in response to HS or exogenous ABA treatment, stratified WT Col-0 seeds were germinated on MSO at 37℃ or on MS-ABA5 medium (MSO + 5 μM ABA) at 22℃. Total RNA was isolated from imbibed seeds at various time points using TRIzol reagent (Invitrogen, USA). Complementary DNA was synthesized using SuperScript II RNase H-reverse transcriptase (Thermo Fisher Scientific, USA), and RT-qPCR analysis was performed using *AtCYS5*-specific primers (P1 forward and P2 reverse; Supplementary Fig. 1A) as described by Hwang et al. (2010). Relative *AtCYS5* transcript levels were determined using ImageJ software (http://rsb.info.nih.gov/ij).

Histochemical GUS staining and fluorometric GUS assays

To monitor the activity of the *AtCYS5* promoter during seed germination, a fragment of *AtCYS5* (-1542 to +58 bp relative to transcriptional start site) encompassing the promoter region of *AtCYS5* (-1542 to -1), the 5'-untranslated region (+1 to +31), and the translational start site ATG with nine amino acids (+32 to +58) was obtained by PCR amplification of *Arabidopsis* genomic DNA using primers *AtCYS5P*-F1 (5'-AAGCTTATGGAACCGTGAGTGTGATGATGGC-3') and *AtCYS5P*-R1 (5'-ACCCGGGGAAGAAGGAAGACGACCTTACTA GTC-3'). PCR amplicons were cloned into the vector pGEM-T Easy (Promega, USA) and sequenced to confirm the fidelity of amplification (Supplementary Fig. 2). The putative *AtCYS5* promoter sequence was excised from the pGEM-T Easy vector with *Hin*dIII/*Bam*HI and subcloned into the same sites of pCambia1381 (Marker Gene Technol., USA). The respective construct (P_{AtCYS5}::*GUS*) was introduced into *Agrobacterium tumefaciens* strain GV3101, which was used to produce transgenic *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998).

GUS activity in transgenic *Arabidopsis* plants was analyzed by histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; Duchefa, The Netherlands) as described by Jefferson et al. (1987). Imbibed seeds were harvested and immediately fixed for 30 min in ice-cold 90% acetone (Vanderbeld and Snedden

2007), rinsed with water, and incubated in 50 mM sodium phosphate buffer (pH 7.0), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 0.2% Triton X-100 containing 1 mM X-gluc. The histochemical reaction was performed in the dark at 37℃ for 12 h, after which the samples were transferred to 70% ethanol to remove the chlorophyll. Digital images were obtained under a stereoscope (Olympus SZX12, Japan).

Quantitative measurement of GUS activity in protein extracts was performed using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (4-MUG; Sigma-Aldrich, USA). Protein extracts were isolated by grinding the tissues in extraction buffer (50 mM sodium phosphate [pH 7.0], 10 mM EDTA, 0.1% SDS, 0.1% Triton X-100, 10 mM B-mercaptoethanol, and 25 μg/ml phenylmethylsulfonyl fluoride), followed by centrifugation (12,000 rpm for 10 min). The supernatants were combined with extraction buffer containing 0.8 mM 4-MUG, and GUS activity at various time points was determined in triplicate and calculated as pmoles 4-methylumbelliferone (4-MU)/min/mg protein as described by Chen et al. (2010).

Generation of *AtCYS5*-overexpressors

To investigate the effect of *AtCYS5* on seed germination and seedling growth *in vivo*, *AtCYS5*-overexpressing transgenic lines were generated as described by Song et al. (2014). *AtCYS5* cDNA was isolated from 2-day-old Col-0 seedlings by RT-PCR using primers *AtCYS5* P1 forward and P2 reverse (Supplementary Fig. 1A). PCR amplicons were cloned into the pGEM-T Easy vector (Promega) and sequenced to confirm the fidelity of amplification. The *AtCYS5* cDNA was excised from the pGEM-T Easy vector with *Xba*I/*Kpn*I and subcloned into the same sites of pCambia1301 (Marker Gene Technol.). The respective construct (*35S:AtCYS5*) was introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* plants as previously described (Clough and Bent, 1998). Homozygous T_3 generation plants were obtained as described by Koo et al. (2002).

To examine the inhibitory activity of AtCYS5 in *AtCYS5*-overexpressors, CP activity measurements in *AtCYS5*-overexpressors were performed as described by Hong et al. (2007).

Germination and seedling growth measurements

AtCYS5-overexpressors, an *AtCYS5* knockout line (*cys5*), and untransformed WT seeds were collected from fully mature siliques of dehydrated plants of the same age for seed germination and seedling growth assays (Je et al., 2014). Stratified seeds (>100 seeds per replicate) were germinated on MSO medium containing 2% sucrose, and the plates were transferred to a growth chamber (EYELA, Japan) at 22 or 50℃ and incubated for various periods of time. Germination was scored by microscopy based on radical emergence (Hwang et al., 2009).

To measure seedling growth, stratified seeds treated with HS at 50℃ for 115 min or normal temperature conditions (22℃) were germinated for 7 days prior to fresh weight and primary root length measurements.

To compare the effects of ABA on germination and seedling growth in various plants, seeds from *AtCYS5*-overexpressors, *cys5*, and WT were germinated on MS medium with or without 0.5 or 0.7 μM ABA (Sigma). The plates were transferred to a growth chamber set 22℃. Germination rates at various time points and seedling growth at 7 days were scored as described above.

Transient promoter activation assay

Transcriptional activators of the *AtCYS5* promoter were identified using the reporter plasmid construct P_{AtCYS5}:*GUS*. The effector plasmids used in the assay contained *ABF1*, *ABF2*, *ABF3*, *ABF4*, *ABI5*, or *AREB3* cDNAs fused in-frame at their C-termini to the green fluorescent protein (*GFP*) gene (Je et al., 2014). Each effector chimeric gene was inserted between the *CaMV35S* promoter and the nopaline synthase terminator in pCambia1381 (Marker Gene Technol.) for constitutive expression of the cDNA (Fig. 5A). A pJD300 plasmid expressing luciferase (*LUC*) driven by the *CaMV35S* promoter was used for normalization of gene expression in samples with different transformation efficiencies (Park et al., 2004). The reporter and effector plasmids were introduced into *Arabidopsis* leaf protoplasts (Je et al., 2014), and GUS activity was analyzed as described by Jefferson et al. (1987).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described by Je et al. (2014), using double-stranded (ds)-oligonucleotide probes corresponding to a fragment of the *AtCYS5* promoter spanning the G-box (*wG*; CACGTG) and two C-box (*wC1* and *wC2*; CACGTC) sequences (Fig. 6A). The ABRE CACGT(G/C)GC represents a subset of the G- and C-box sequences (Kim et al., 2004). The sequences of the six oligonucleotide probes used in EMSA are as follows: WT C1 and G-box (*wC1+wG*) and WT C-box 2 (*wC2*), which correspond to the native promoter fragments, with the sequences 5'-CTGCCACACGTCATGC CACGTGGCGGAC-3' for *wC1+wG* (*wC1* [right side] and *wG* [left side] sequences are underlined) and 5'-CATAC CATACCACGTCATTGTCCAG-3' for *wC2* (*wC2* sequence is underlined); *mG*, 5'-CTGCCACACGTCATGCactacGGCGGAC-3' (mutations in *wG* are indicated by lowercase letters); *mC1*, 5'-CTGCCAtgtagCATGCCACGTGGCGGAC-3' (mutations in *wC1* are indicated by lowercase letters); *mC1+mG*, 5'-CTGCCAtgtagCATGCactacGGCGGAC-3' (mutations in *wC1+wG* are indicated by lowercase letters); and *mC2*, 5'-CATACCATACtgtagCATTGTCCAG-3' (mutations in *wC2* are indicated by lowercase letters). ^{32}P-labeling of probe and purification of recombinant proteins were carried out as described by Lim et al. (2007).

RESULTS AND DISCUSSION

AtCYS5 transcript levels increase during seed germination

To determine whether AtCYS5 might also function in HS responses, we isolated total RNA from germinating *Arabidopsis* seeds under HS conditions. *AtCYS5* transcript levels peaked at 12 h in stratified seeds imbibed under both normal (22℃) and HS conditions (37℃) (Fig. 1A). However, at 22℃, *AtCYS5* transcript levels sharply decreased after 12 h,

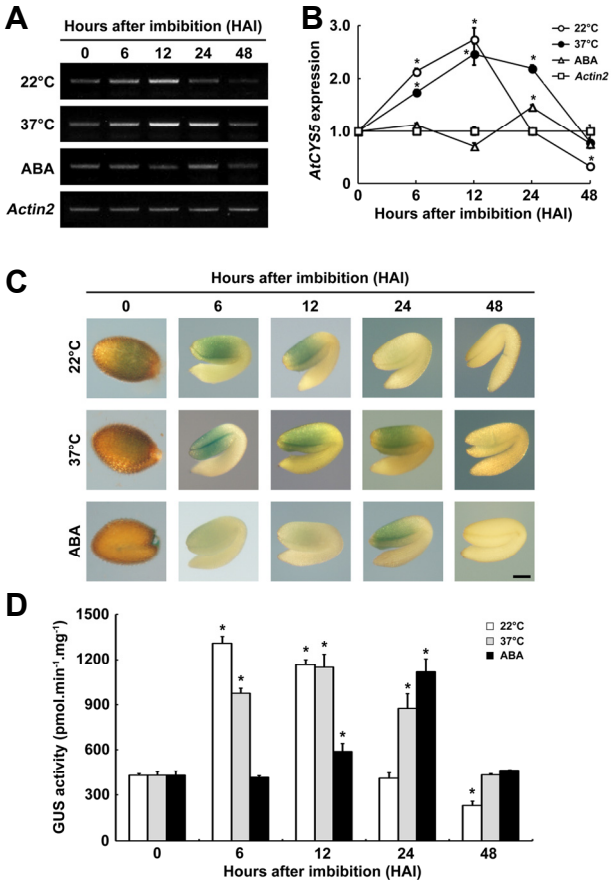

Fig. 1. *AtCYS5* expression in imbibed seeds increases under mild heat stress and exogenous ABA treatment. (A) Imbibed wild-type Col-0 *Arabidopsis* seeds were germinated at 22℃ and 37℃ and exposed to 5.0 μM ABA (under 22℃) for the indicated time periods. RT-qPCR analysis (30 cycles) was performed on total RNA; ethidium bromide-stained gels are shown. *Actin2* (At3g18780) transcript levels are shown as the loading control. (B) Quantitative analysis of signal intensity in the ethidium bromide-stained gels shown in (A), as measured with ImageJ software. Values represent mean ± SD, and values prior to treatment (0 h) were set at 1. Statistically significant differences between the exposure times are indicated by asterisks ($n = 3$; * $P < 0.01$ by Student's *t*-test). (C) Histochemical localization of GUS expression in transgenic *Arabidopsis* plants carrying the *GUS* coding region fused to the *AtCYS5* promoter (P_{AtCYS5}:*GUS*). Seeds were germinated at 22℃ and 37℃ and exposed to 1.0 μM ABA (at 22℃) for the indicated periods of time. Scale bar is 100 μm. (D) GUS activity over time was determined by quantitative fluorometric assays using germinated seeds. The effect of mild heat shock or exogenous ABA treatment was investigated in seeds. The data presented are the mean ± SD of three independent experiments (* $P < 0.01$ by Student's *t*-test).

returned to control (0 h) levels at 24 h, and were barely detected at 48 h, whereas at 37℃, *AtCYS5* transcript levels gradually decreased after 12 h but were 2.5-fold higher at 24 h than under normal growth conditions (Fig. 1B). Therefore, *AtCYS5* is induced during germination and its expression is sustained under HS, suggesting that AtCYS5 likely functions in the maintenance of germination under HS conditions.

If AtCYS5 is required for germination, *AtCYS5* transcript levels should increase during seed germination in medium containing a germination inhibitor. To investigate this notion, we measured *AtCYS5* transcript levels in seeds in the presence of exogenous ABA. As shown in Fig. 1A and 1B, when germination was blocked by ABA treatment, the expression of *AtCYS5* did not significantly change during processing time. However, at 24 h (when untreated seeds germinated), *AtCYS5* transcript levels were slightly higher than at 0 h and they returned to basal levels at 48 h. Therefore, as determined by RT-qPCR, *AtCYS5* contributes to seed germination by helping the plant overcome germination inhibitory factors such as HS and ABA.

To determine whether increased *AtCYS5* transcript levels correspond to changes in *AtCYS5* promoter activity *in vivo*, we compared *AtCYS5* promoter activity in P_{AtCYS5}:*GUS* transgenic plants with or without HS or ABA treatment to the *AtCYS5* expression levels obtained by RT-qPCR (Fig. 1C). Like the expression patterns obtained by RT-qPCR, under normal growth conditions, GUS activity increased within 6 h of imbibition but was nearly undetectable at 24 and 48 h. However, under HS conditions, GUS expression continued to be detected at 24 h of treatment. Additionally, *AtCYS5* expression in ABA-treated seeds peaked at 24 h and was nearly undetectable at 48 h. The *GUS* reporter gene driven by the *AtCYS5* promoter was primarily expressed in cotyledons. The GUS activity patterns mirrored the GUS staining patterns under the same conditions (Fig. 1D). These results indicate that *AtCYS5* expression is triggered just prior to germination, and they suggest that AtCYS5 might play an important role in helping plants overcome germination inhibitory factors during seed germination.

Overexpression of *AtCYS5* increases seed germination and seedling growth

To confirm the function of AtCYS5 in seed germination and seedling growth *in vivo*, we created *AtCYS5* overexpression transgenic lines (*35S:AtCYS5*; Supplementary Fig. 1B). To determine the copy numbers of *AtCYS5* in the transgenic plants, T_1 plants were self-pollinated and the progeny (T_2) were allowed to segregate on selection medium. Following self-pollination of the T_2 lines, we selected two T_3 homozygous lines (*35S:AtCYS5*-L6 and -L7) containing a single insertion (Supplementary Fig. 1C). In addition, we analyzed the *in vivo* function of *AtCYS5* using a T-DNA insertional mutant (*cys5*) with a disrupted *AtCYS5* locus. Analysis of the sequence of the T-DNA flanking region in *cys5* indicated that the insertion was in the exon of *AtCYS5* (Supplementary Fig. 1A). PCR amplicons encompassing the T-DNA insertion site were not detected for the *cys5* mutant (Supplementary Fig. 1C); however, when the P2 primer and the T-DNA primer (P4) were used, amplicons were detected (Supplementary Fig. 1D). PCR genotyping revealed that the *cys5* mutation resulted in the complete loss of *AtCYS5* expression. Accordingly, the endogenous CP inhibitory activity of L6 and L7 was

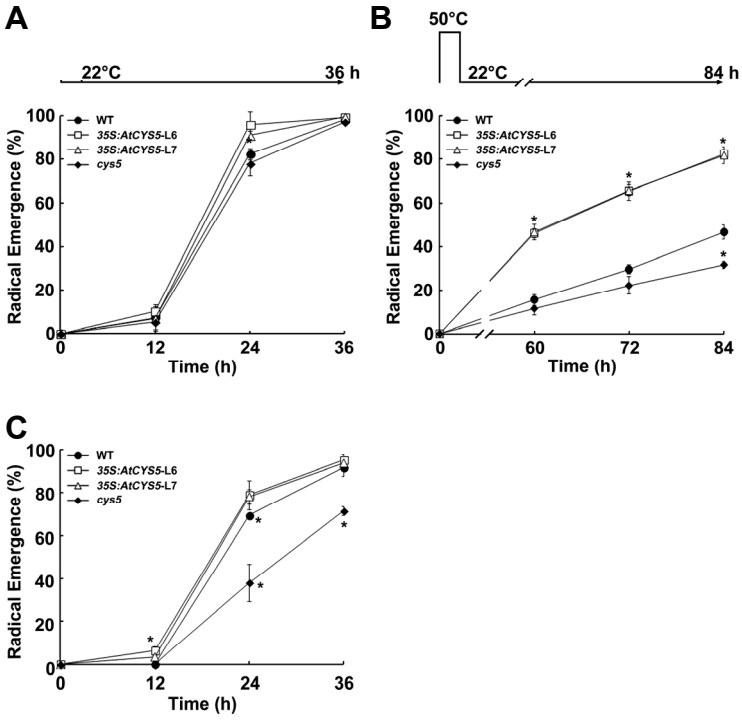

Fig. 2. Comparison of seed germination rates in transgenic *AtCYS5* plants. Time course of germination (in hours after imbibition) for freshly harvested seeds of untransformed wild-type (WT), *AtCYS5* overexpression lines (*35S:AtCYS5*-L6, *35S:AtCYS5*-L7), and *AtCYS5* knockout line (*cys5*). (A) Seeds were surface-sterilized, plated on MSO medium, and stratified in the dark at 4℃ for 3 d before shifting the plates to a growth chamber. Percent radical emergence was measured at 12 h intervals after shifting to a growth chamber set at 22℃. (B) Imbibed seeds were treated at 50℃ for 115 min as shown and shifted to a growth chamber set at 22℃. (C) Imbibed seeds were plated on MSO containing 1.0 μM ABA and stratified in the dark at 4℃ for 3 d before shifting the plates to a growth chamber set at 22℃. Bars represent mean ± SD (n = 3, $*P < 0.01$ by Student's t-test).

higher than that of WT and *cys5* (Supplementary Fig. 1E).

We imbibed seeds from the two independent *AtCYS5* overexpression lines, the knockout mutant, and untransformed WT, stratified them for 3 d in the dark at 4℃, and allowed them to germinate under long-day conditions at 22℃ and 50℃ to investigate the effect of temperature on germination. Under normal growth conditions (22℃), we detected little difference among the four lines (Fig. 2A). Approximately 20% lower germination rates were observed in WT and *cys5* seeds at 24 h after imbibition compared to L6 and L7, but by 36 h, the germination rate of all four lines was 100%. The similarity of phenotype to WT in *cys5* may be due to functional redundancy of other *AtCYS*s in *Arabidopsis* genome. However, under HS conditions, the germination rate was strongly reduced in WT and *cys5* seeds, with only 30-50% of the seeds having germinated by 84 h compared to 80% in the *35S:AtCYS5* transgenic lines (Fig. 2B). Therefore, the increased germination rate in *35S:AtCYS5* transgenic seeds indicates that AtCYS5 helps improve germination under HS conditions.

To investigate the effect of ABA, an inhibitor of seed germination, on *35S:AtCYS5* seed germination, we incubated WT and transgenic seeds on MS medium containing 1.0 μM ABA (Fig. 2C). At 22℃, there was no significant difference in germination rate between WT and *35S:AtCYS5* transgenic seeds, whereas the germination rate was lower in *cys5*, with 70% of the seeds having germinated by 36 h compared to 100% for WT and *35S:AtCYS5* seeds. Collectively, these results strongly suggest that AtCYS5 is involved in promoting germination under HS and ABA treatment.

Overexpressing *AtCYS5* improves HS tolerance during post-germination growth

Since seeds of the *AtCYS5* overexpressors exhibited increased germination under HS compared to WT, we investigated whether the overexpression of *AtCYS5* is associated with increased HS tolerance during post-germination as well. We compared two morphological phenotypes (fresh weight and primary root length) in WT and *AtCYS5* transgenic *Arabidopsis* plants, including an *AtCYS5* overexpressor and a knockout mutant, by subjecting imbibed seeds to HS for 115 min and observing the plants after a 7 day recovery period at 22℃ (Fig. 3). Under normal growth conditions (22℃), the *AtCYS5* transgenic plants exhibited no difference in morphology or growth compared to WT. However, while the growth of both WT and the transformants was inhibited under HS conditions, this inhibition was much more severe in WT and *cys5* than in the *AtCYS5* overexpressors (Fig. 3A). For example, under HS conditions, the fresh weight was reduced by approximately 55% for the overexpressors and 95% for WT. The reduction in fresh weight was much more severe in *cys5* than in WT (Fig. 3B). Primary root growth was altered in a similar manner (Fig. 3C). Measurements of fresh weight and root length, which reflect the level of damage due to HS, indicated that seedling growth was reduced in plants from HS-exposed seeds compared to the untreated controls in all lines examined. However, the *AtCYS5* overexpression lines had higher fresh weights and longer roots than WT or *cys5* under HS (Figs. 3B and 3C). These results indicate that *AtCYS5* overexpression increased the resistance to HS during the post-germination period.

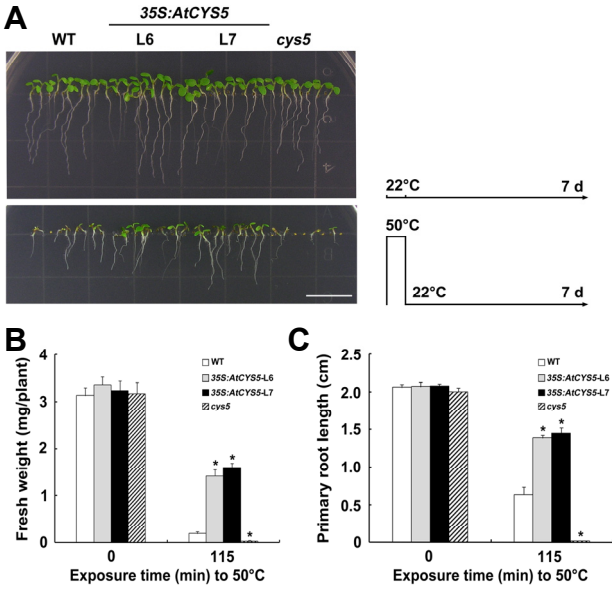

Fig. 3. Comparison of seedling growth in transgenic *AtCYS5* plants. Phenotypes are shown for 7-day-old untransformed wild-type (WT), *AtCYS5* overexpression lines (*35S:AtCYS5*-L6, *35S:AtCYS5*-L7), and *AtCYS5* knockout line (*cys5*). (A) Imbibed seeds were treated at 50℃ for 115 min, and photographs were taken after 7 days of recovery at 22℃. Scale bar is 1 cm. (B) Fresh weights of 7-day-old WT and transgenic seedlings. (C) Quantitative analysis of primary root lengths of 7-day-old seedlings from each transgenic line. Values represent mean ± SD ($n = 3$, $*P < 0.01$ by Student's *t*-test).

Overexpression of *AtCYS5* accelerates post-germination growth in plants from ABA-treated seeds

HS increases ABA levels in plant cells (Toh et al., 2008). Therefore, the increased germination in *35S:AtCYS5* transgenic seeds under HS conditions might have been due to the insensitivity of the seeds to increased ABA levels. We reasoned that overexpression of *AtCYS5* may also lead to increased post-germination growth under ABA treatment. To test this hypothesis, surface-sterilized WT, *35S:AtCYS5*, and *cys5* seeds were plated on medium supplemented with 0.5 or 0.7 μM ABA, imbibed, stratified, and allowed to germinate, and the plants were photographed 7 days later. In the absence of ABA, the *AtCYS5* transgenic plants exhibited no difference in morphology or growth compared to WT. However, as the concentration of ABA increased, the growth inhibition became more severe in both WT and transgenic plants, but this effect was much more severe in WT and *cys5* than in the *AtCYS5* overexpressors (Fig. 4A). For example, the fresh weight of the overexpressors was reduced by approximately 55% in response to 0.5 μM ABA and 70% in response to 0.7 μM ABA treatment. By contrast, in WT, the fresh weight was reduced by 90% and 98% in response to 0.5 and 0.7 μM ABA treatment, respectively (Fig. 4B). The reduction in fresh weight was much more severe in *cys5* than in WT. Primary root growth was altered in a similar manner (Fig. 4C). Measurements of fresh weight and root length showed that seedling growth was proportional to the ABA concentration in the medium for all lines but was 2-5 fold higher in the *35S:AtCYS5* transgenic lines than in WT or *cys5* (Figs. 4B and 4C). These results suggest that AtCYS5

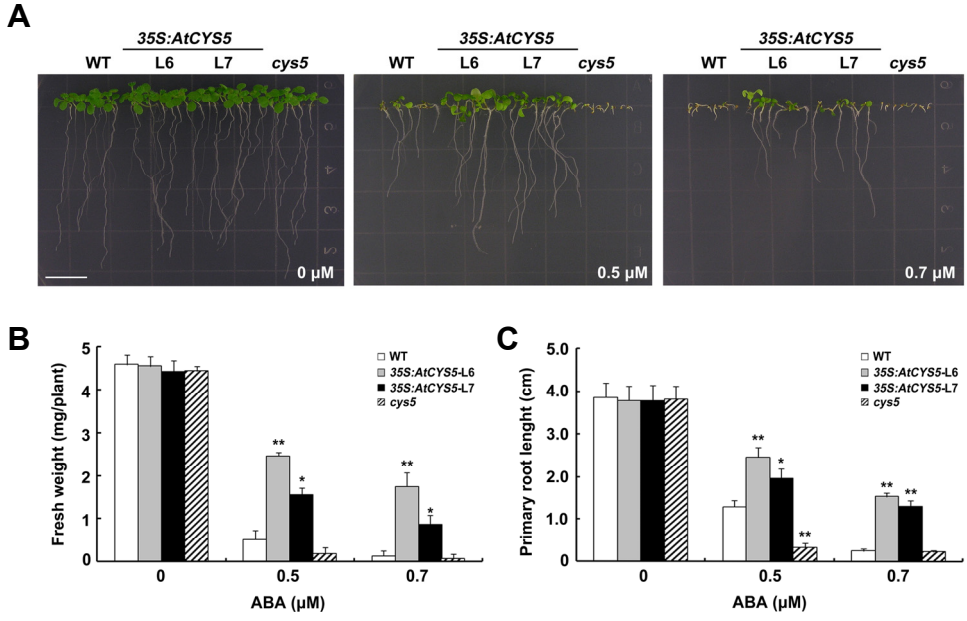

Fig. 4. Seedling growth of transgenic *AtCYS5* plants in response to ABA. (A) Seeds of untransformed wild-type (WT), two independent *35S:AtCYS5* lines (*35S:AtCYS5*-L6, -L7), and the *AtCYS5* knockout line (*cys5*) were plated on MSO agar medium containing 0.5 μM (middle panel) or 0.7 μM ABA (right panel). The photographs were taken 7 days after sowing. Scale bar is 1 cm. (B) Fresh weights of 10-day-old WT and transgenic seedlings. (C) Quantitative analysis of primary root length in 10-day-old seedlings from each transgenic line. Values represent mean ± SD ($n = 3$, $*P < 0.05$, $**P < 0.01$ by Student's *t*-test).

reduces the sensitivity of plants to ABA during seed germination and post-germination growth.

ABF1 and ABF3 transactivate *AtCYS5* expression in *Arabidopsis* protoplasts

ABA- and stress-responsive *cis*-acting elements are usually found in the promoter regions of stress-inducible genes (Zhang et al., 2005). Thus, we analyzed the upstream region (-1542 bp upstream of the transcription start site) of *AtCYS5* to identify putative ABA- or HS-responsive *cis*-acting elements. The *AtCYS5* promoter contains three putative ACGT-containing ABREs identical to the well-characterized motifs present in inducible promoters that respond to ABA and/or abiotic stress (Zhang et al., 2005), whereas no HS element (HSE) was identified (Supplementary Fig. 2). The induction of gene expression by HS does not always require a HSE (Je et al., 2014). For example, *Arabidopsis* ABRE sequences (CCACGTGG) are important for transcriptional activation in response to a variety of stress conditions, including heat (Je et al., 2014; Toh et al., 2008). Therefore, the absence of a HSE in the *AtCYS5* promoter suggests that some other *cis*-elements in the promoter, such as ABREs, along with their cognate binding proteins, might be involved in the thermoregulation of *AtCYS5* expression.

ABREs represent a subset of the C- and G-box sequences [CACGT(C/G)] present in the promoter regions of many light-regulated genes (Giuliano et al., 1988). The *AtCYS5* promoter contains three putative ABREs containing C- and G-boxes (Supplementary Fig. 2). C- and G-box-containing ABREs are the binding sites for the ABFs, ABA-insensitive 5 (ABI5), and AREBs, i.e., group A bZIP transcription factors involved in ABA-dependent signaling in *Arabidopsis* (Jakoby et al., 2002). Thus, we focused on members of these transcription factor families to explore the importance of the ABREs in the *AtCYS5* promoter. We evaluated the ability of bZIPs to regulate *AtCYS5* transcription via these motifs by performing transient promoter activation assays in *Arabidopsis* leaf protoplasts. Effector constructs were designed for constitutive overexpression of each full-length ABF, ABI5 and AREB3, whereas the reporter construct consisted of the *AtCYS5* promoter (-1542 to +58 bp; Supplementary Fig. 2) fused upstream of GUS (P_{AtCYS5}:*GUS*). The pJD300 plasmid was used to nomalize effects arising from variations in transformation efficiency between samples (Park et al., 2004). Both constructs were mixed and co-transformed into *Arabidopsis* leaf protoplasts (Fig. 5A). Co-expression of the transcription factors did not transactivate the expression of the *GUS* reporter gene, whereas the addition of ABA increased GUS/LUC activity approximately 5- to 7-fold, compared to ABA-treated samples that received no effector plasmid (Fig. 5B). These results suggest that bZIP transcription factors function as transcriptional activators of *AtCYS5* expression in the presence of ABA and that the overproduction of bZIP factors alone is not sufficient to induce the expression of *AtCYS5*.

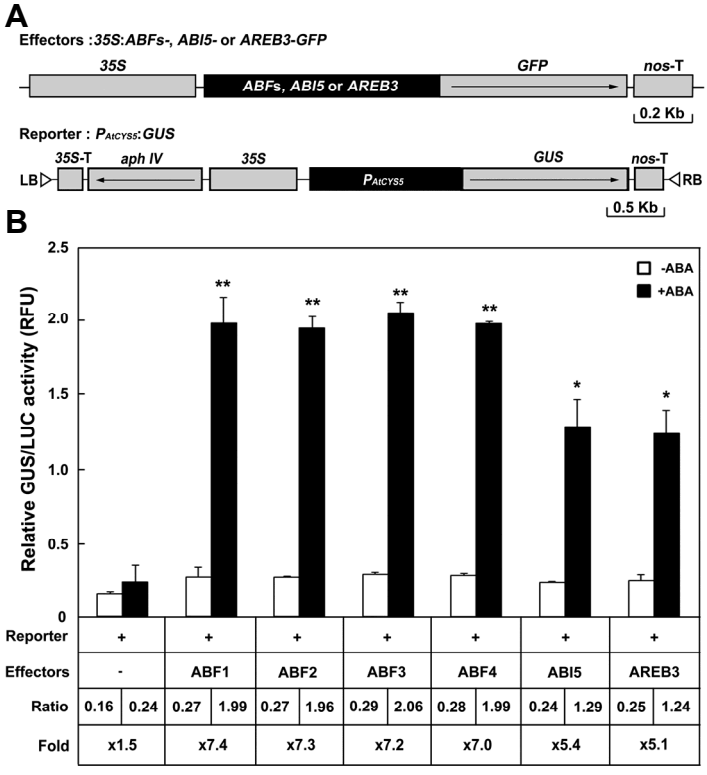

Fig. 5. Group A bZIPs activate the transcription of *AtCYS5* in the presence of ABA. (A) Schematic representation of the effector and reporter constructs used for the transient promoter activation assays. The effector constructs used to express the bZIP factor tagged with green fluorescent protein (GFP) contained the *CaMV35S* promoter (*35S*) fused to the entire ORF of the test proteins (except the stop codon), followed by the nopaline synthase gene terminator (*nos*-T). The bZIP cDNAs encoded ABF1, 2, 3, and 4, ABI5, and AREB3. The reporter construct (P_{AtCYS5}:*GUS*) contained the *GUS* gene inserted between the *AtCYS5* promoter (P_{AtCYS5}) and *nos*-T, as well as the *CaMV35S* promoter (*35S*), bacterial *hygromycin B phosphotransferase IV* (*aph IV*), and a *35S* gene terminator (*35S*-T) cassette to provide a selectable marker for *Arabidopsis* transformation. LB and RB indicate left and right T-DNA borders, respectively. (B) Histogram showing GUS/LUC activity in transformants harboring the reporter construct with the effector construct, as indicated. Solid bars indicate protoplasts treated with ABA and white bars indicate protoplasts not treated with ABA. Fold represents the fold-increase in GUS/LUC activity in transformants harboring both reporter and effector compared to the GUS/LUC activity in transformants in the absence of ABA. Bars represent mean ± SD (n = 3, $*P < 0.05$, $**P < 0.01$ by Student's t-test).

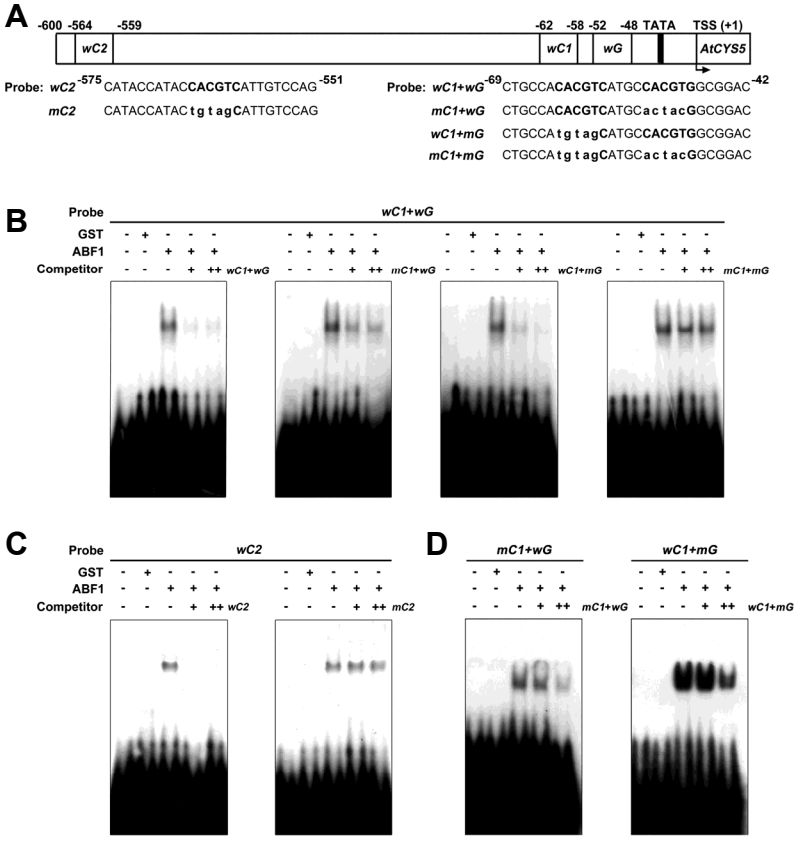

Fig. 6. EMSA of the ABRE-binding activity of ABF1. (A) Schematic diagram of the *AtCYS5* promoter driven by a 0.6 kb sequence upstream of the *AtCYS5* transcription start site (TSS, +1) containing three wild-type ABREs (*wG*, *wC1*, and *wC2*). Probes show sequences of the oligonucleotides used in EMSA. Mutant *G* (*mG*), *C1* (*mC1*), and *C2* (*mC2*) are oligonucleotides containing the mutations indicated in lowercase letters. Solid box represents the TATA-box. (B) EMSA of ABRE-binding activity of ABF1. ^{32}P-labeled *wC1+wG* was used as a probe, and unlabeled *wC1+wG*, *mC1+wG*, *wC1+mG*, and *mC1+mG* fragments were used as competitors. (C) ^{32}P-labeled *wC2* was used as a probe, and unlabeled *wC2* and *mC2* fragments were used as competitors. (D) ^{32}P-labeled *mC1+wG* and *wC1+mG* were used as probes, and unlabeled *mC1+wG* and *wC1+mG* fragments were used as competitors. Shown are autoradiograms of gels used to analyze the binding reactions of the indicated composition, where minus (-) indicates omission, plus (+) indicates addition, and "++" indicates twice the amount of a component compared to +.

ABF1 and ABF3 bind directly to the *AtCYS5* promoter

Since in the presence of ABA, the expression of *AtCYS5* was activated by bZIP transcription factors in the transient promoter activation assays (Fig. 5), we compared the ability of bZIP factors to physically bind to the C- and G-boxes in the *AtCYS5* promoter via EMSA (Fig. 6A). When ^{32}P-labeled oligonucleotide probes (*wC1+wG* or *wC2*) corresponding to the native *AtCYS5* promoter fragment were incubated with GST alone (control) or purified recombinant GST-bZIP fusion proteins, bands with retarded mobility were observed in the EMSA only for GST-ABF1 and GST-ABF3 (Fig. 6 and 7), indicating that ABF1 and ABF3 physically bound to both the *wC1+wG* and *wC2* probes. By contrast, ABF2, ABF4, ABI5, and AREB3 did not bind to C- or G-box in the *AtCYS5* promoter (Supplementary Fig. 3). The addition of unlabeled *wC1+wG* or *wC2* to the ^{32}P-*wC1+wG* or ^{32}P-*wC2* binding reaction reduced binding of ABF1 to the probes in competition assays (Fig. 6B and 6C). The addition of unlabeled competitor *mC1+wG* or *wC1+mG* to the ^{32}P-*wC1+wG* binding reaction reduced the binding of ABF1 to the probes to a similar degree (Fig. 6B), indicating that ABF1 binds equally to *wC1* and *wG* (Fig. 6C). The addition of unlabeled *mC1+mG* and *mC2* did not reduce the binding of ^{32}P-*wC1+wG* or ^{32}P-*wC1*. Interestingly, the signal generated by the binding of ABF1 to *wC1* was stronger than that using *wG*, providing evidence that ABF1 binds more efficiently to the C-box than the G-box (Fig. 6D).

In the case of ABF3, the addition of unlabeled *wC1+wG* or *wC2* to the ^{32}P-*wC1+wG* or ^{32}P-*wC2* binding reaction reduced binding of ABF3 to the probes (Figs. 7A and 7B). Unlike for ABF1, the addition of unlabeled competitor *mC1+wG* to the ^{32}P-*wC1+wG* reaction did not reduce the binding of ABF3 to ^{32}P-*wC1+wG*, but the addition of competitor *wC1+mG* to ^{32}P-*wC1+wG* reduced the binding of ABF3 to the probes (Fig. 7A). These results indicate that ABF3 binds strongly to *wC1* but very weakly to *wG*. The addition of unlabeled *mC2* to the ^{32}P-*wC2* binding reaction did not reduced the binding of ABF3 to the probes (Fig. 7B), indicating that ABF3 can also bind to *wC2*. The signal generated by the binding of ABF3 to *wG* was stronger than that with *wC1*, suggesting that ABF3 binds more efficiently to the G-box than the C-box (Fig. 7C). Finally, the binding activity of *wC2* was between that of *wG* and *wC1* (Figs. 7B and 7C). These observations indicate that ABF1 and ABF3 bind to the *C2*, *C1*, and *G* regions of the *AtCYS5* promoter but with differential binding abilities.

In conclusion, group A bZIPs ABF1 and ABF3 regulate *AtCYS5* expression through the ABRE-mediated signaling pathway in the presence of ABA. The upregulated expression of *AtCYS5* increases thermotolerance during seed germination and seedling growth. These findings suggest that *AtCYS5* might be useful in molecular breeding aimed at increasing plant tolerance to HS during seed germination and post-germination growth.

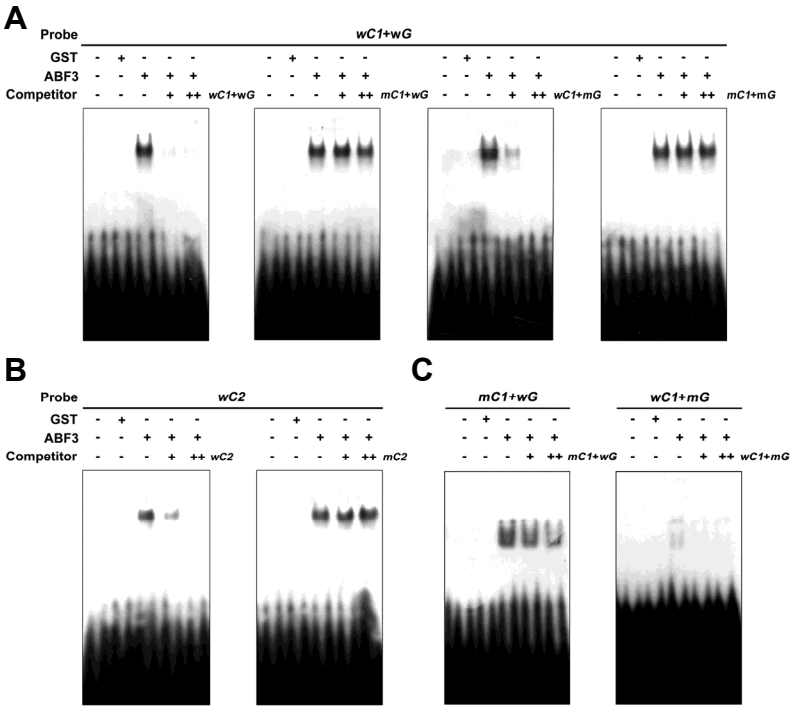

Fig. 7. EMSA of ABRE-binding activity of ABF3. (A) ^{32}P-labeled *wC1+wG* was used as a probe, and unlabeled *wC1+wG*, *mC1+wG*, *wC1+mG*, and *mC1+mG* fragments were used as competitors. (B) ^{32}P-labeled *wC2* was used as a probe, and unlabeled *wC2* and *mC2* fragments were used as competitors. (C) ^{32}P-labeled *wC1+mG* and *mC1+wG* were used as probes, and unlabeled *wC1+mG* and *mC1+wG* fragments were used as competitors. Shown are autoradiograms of gels used to analyze binding reactions of the indicated composition, where minus (-) indicates omission, plus (+) indicates addition, and "++" indicates twice the amount of a component compared to +.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by the Next-Generation BioGreen 21 Program (SSAC, grant no. PJ01107101, Rural Development Administration, Republic of Korea) and a National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (2017R1A2B1001852).

REFERENCES

Bae, E.K., Lee, H., Lee, J.S., and Noh, E.W. (2010). Isolation and characterization of osmotic stress-induced genes in poplar cells by suppression subtractive hybridization and cDNA microarray analysis. Plant Physiol. Biochem. *48*, 136-141.

Chen, H., Hwang, J.E., Lim, C.J., Kim, D.Y., Lee, S.Y., and Lim, C.O. (2010). *Arabidopsis* DREB2C functions as a transcriptional activator of *HsfA3* during the heat stress response. Biochem. Biophys. Res. Commun. *401*, 238-244.

Christoff, A.P., Passaia, G., Salvati, C., Alves-Ferreira, M., Margis-Pinheiro, M., and Margis, R. (2016). Rice bifunctional phytocystatin is a dual molulator of legumain and papain-like proteases. Plant Mol. Biol. *92*, 193-207.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. *16*, 735-743.

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2005). AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell *17*, 3470-3488.

Gabriele, S., Rizza, A., Martone, J., Circelli, P., Costantino, P., and Vittorioso, P. (2010). The Dof protein DAG1 mediates PIL5 activity on seed germination by negatively regulating GA biosynthetic gene AtGA3ox1. Plant J. *61*, 312-323.

Giuliano, G., Pichersky, E., Malik, V.S., Timko, M.P., Scolnik, P.A., and Cashmore, A.R. (1988). An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. Proc. Natl. Acad. Sci. USA *85*, 7089-7093.

Hong, J.K., Hwang, J.E., Lim, C.J., Yang, K.A., Jin, Z.L., Kim, C.Y., Koo, J.C., Chung, W.S., Lee, K.O., Lee, S.Y., et al. (2007). Over-expression of Chinese cabbage phytocystatin 1 retards seed germination in *Arabidopsis*. Plant Sci. *172*, 556-563.

Huang, B., and Xu, C. (2008). Identification and characterization of proteins associated with plant tolerance to heat stress. J. Intergr. Plant Biol. *50*, 1230-1237.

Hwang, J.E., Hong, J.K., Je, J., Lee, K.O., Kim, D.Y., Lee, S.Y., and Lim, C.O. (2009). Regulation of seed germination and seedling growth by an *Arabidopsis* phytocystatin isoform, *AtCYS6*. Plant Cell Rep. *28*, 1623-1632.

Hwang, J.E., Hong, J.K., Lim, C.J., Chen, H., Je. J., Yang, K.A., Kim, D.Y., Choi, Y.J., Lee, S.Y., and Lim, C.O. (2010). Distinct expression patterns of two *Arabidopsis* phytocystatin genes, *AtCYS1* and *AtCYS2,* during development and abiotic stresses. Plant Cell Rep. *29*, 905-915.

Jakoby, M., Weisshaar, B., Dröge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroi, T., and Parcy, F. (2002). bZIP transcription factors in Arabidopsis. Trends Plant Sci. *7*, 106-111.

Je, J., Song, C., Hwang, J.E., Chung, W.S., and Lim, C.O. (2014) DREB2C acts as a transcriptional activator of the thermo tolerance-related *phytocystatin 4* (*AtCYS4*) gene. Transgenic Res. *23*, 109-123.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: *β*-glucuronidase as a sensitive and versatile gene fusion

marker in higher plants. EMBO J. *6*, 3901-3907.

Julián, I., Gandullo, J., Santos-Silva, L.K., Diaz, I., and Martinez, M. (2013). Phylogenetically distant barley legumains have a role in both seed and vegetative tissues. J. Exp. Bot. *64*, 2929-2941.

Kidrič, M., Kos, J., and Sabotič, J. (2014). Proteases and their endogenous inhibitors in the plant to abiotic stress. Bot. Serb. *38*, 139-158.

Kim, S., Kang, J., Cho, D., Park, J.H., and Kim, S.Y. (2004). ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its over expression affects multiple stress tolerance. Plant J. *40*, 75-87.

Koo, J.C., Chun, H.J., Park, H.C., Kim, M.C., Koo, Y.D., Koo, S.C., Ok, H.M., Park, S.J., Lee, S.H., Yun, D.J., et al. (2002). Over-expression of a seed specific hevein-like antimicrobial peptide from *Pharbitis nil* enhances resistance to a fungal pathogen in transgenic tobacco plants. Plant Mol. Biol. *50*, 441-452.

Lim, C.J., Hwang, .JE., Chen, H., Hong, J.K., Yang, K.A., Choi, M.S., Lee, K.O., Chung, W.S., Lee, S.Y., and Lim, C.O. (2007). Over-expression of the *Arabidopsis* DRE/CRT-binding transcription factor *DREB2C* enhances thermotolerance. Biochem. Biophys. Res. Commun. *362*, 431-436.

Martínez, M., Rubio-Somoza, I., Fuentes, R., Lara, P., Carbonero, P., and Díaz, I. (2005). The barley cystatin gene (*Icy*) is regulated by DOF transcription factors in aleurone cells upon germination. J. Exp. Bot. *56*, 547-556.

Park, H.C., Kim, M.L., Kang, Y.H., Jeon, J.M., Yoo, J.H., Kim, M.C., Park, C.Y., Jeong, J.C., Moon, B.C., Lee, J.H., et al., (2004). Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiol. *135*, 2150-2161.

Rodriguez, M.C., Edsgard, D., Hussain, S.S., Alquezar, D. Rasmussen, M., Gilbert, T., Nielsen, B.H., Bartels, D., and Mundy, J. (2010). Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. Plant J. *63*, 212-228.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujida, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., et al., (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J. *31*, 279-292.

Song, C., Je, J., Hong, J.K., and Lim, C.O. (2014). Ectopic expression of an *Arabidopsis* dehydration-responsive element-binding factor *DREB2C* improves salt stress tolerance in crucifers. Plant Cell Rep. *33*, 1239-1254.

Song, C., Chung, W.S., and Lim, C.O. (2016). Overexpression of heat shock factor gene *HsfA3* increases galactinol levels and oxidative stress tolerance in *Arabidopsis*. Mol. Cells *39*, 477-483.

Sun, X., Yang, S., Sun, M., Wang, S., Ding, X., Zhu, D., Ji, W., Cai, H., Zhao, C., Wang, X., and Zhu, Y. (2014). A novel *Glycine soja* cysteine proteinase inhibitor GsCPI14, interacting with the calcium/calmodulin-binding receptor-like kinase GsCBRLK, regulated planttolerance to alkali stress. Plant Mol. Biol. *85*, 33-48.

Szewińska, J., Simińska, J., and Bielawski, W. (2016). The roles of cysteine proteases and phytocystatins in development and germination of cereal seeds. J. Plant Physiol. *207*, 10-21.

Tan, Y., Wei, X., Wang, P., Sun, X., Li, M., and Ma, F. (2016). A phytocystatin gene from *Malus prunifolia* (Willd.) Borkh., *MpCYS5*, confers salt stress tolerance and functions in endoplasmic reticulum stress response in *Arabidopsis*. Plant Mol. Biol. Rep. *34*, 62-75.

Toh, S., Imamura, A., Watanabe, A., Nakabayashi, K., Okamoto, M., Jikumaru, Y., Hanada, A., Aso, Y., Ishiyama, K., Tamura, N., et al. (2008). High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellins action in *Arabidopsis* seeds. Plant Physiol. *146*, 1368-1385.

Vanderbeld, B., and Snedden, W.A. (2007). Developmental and stimulus-induced expression patterns of *Arabidopsis* calmodulin-like genes *CML37*, *CML38* and *CML39*. Plant Mol. Biol. *64*, 683-697.

Van der Hoorn, R.A.L. (2008). Plant proteases: from phenotypes to molecular mechanisms. Annu. Rev. Plant Biol. *59*, 191-223.

Verma, V., Ravindran, P., and Kumar, P. (2016). Plant hormone-mediated regulation of stress responses. BMC Plant Biol. Doi 10.1186/s12870-016-0771-y.

Zakharov, A., Carchilan, M., Stepurina, T., Rotari, V., Wilson, K., and Vaintraub, I. (2004). A comparative study of the role of the major proteinases of germinated common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* (L.) Merrill) seeds in the degradation of their storage proteins. J. Exp. Bot. *55*, 2241-2249.

Zhang, W., Ruan, J., Ho, T.D., You, Y., Yu, T., and Q, R.S. (2005). *Cis*-regulatory element based targeted gene finding: genome-wide identification of abscisci acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. Bioinformatics *21*, 3074-3081.

Zhang, X., Liu, S., and Takano, T. (2008). Two cysteine proteinase inhibitors from *Arabidopsis thaliana*, *AtCYSa* and *AtCYSb*, increasing the salt, drought, oxidation and cold tolerance. Plant Mol. Biol. *68*, 131-143.